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Molecular cloning, expression profiling of adipose triglyceride lipase (ATGL) and forkhead box O1 (FoxO1), and effects of dietary carbohydrate level on their expression in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂)

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ABSTRACT

Adipose triglyceride lipase (ATGL) has been considered as the rate-limiting enzyme involved in lipolysis. In the present study, the ATGL gene and forkhead box O1 (FoxO1), a transcription factor involved in regulation of lipid metabolism, in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) were isolated and characterized, and their gene expression in response to dietary carbohydrate was investigated. Additionally, cell transfection and luciferase assays were used to explore the potential regulation of FoxO1 on the expression of ATGL. The full-length cDNA sequence of ATGL and FoxO1 was 2922 bp and 5471 bp, respectively, encoding 515 amino acid (AA) and 677 AA, respectively. ATGL mRNA was highly expressed in liver, and the expression of FoxO1 was mainly detected in heart and liver. The expression of ATGL decreased significantly in response to high dietary carbohydrate, and the expression of FoxO1 followed the similar pattern with ATGL. The results of dual-luciferase reporter assays showed that grouper ATGL reporter activity was significantly elevated by over-expression of FoxO1, indicating the positive regulation of FoxO1 on ATGL. Meanwhile, insulin significantly repressed the expression of ATGL and FoxO1 in the intraperitoneal injection study. Therefore, the increase of plasma insulin content in the present study may contribute to the accumulation of hepatic triglyceride through down-regulation of FoxO1 and ATGL in transcriptional level. These findings may contribute to further understanding the effects of dietary carbohydrate on lipid metabolism in fish species.

1. Introduction

Non-protein energy source, fat and digestible carbohydrate, has been widely used in aquaculture, mainly due to its benefits on protein retention (Li et al., 2012; Watanabe, 2002; Stone, 2003). However, high-fat diet often leads to abnormal lipid deposition (Du et al., 2008; Lu et al., 2014), and excessive dietary carbohydrate could also aggravate lipid deposition (Peres and Oliva-Teles, 2002; Ye et al., 2009). The abnormal lipid deposition significantly affects the health state of farmed fish, and consequently reduces its harvest yields (Rueda-Jasso et al., 2004; Bolla et al., 2011). Although many studies have been conducted to explore the reason account for lipid deposition caused by high-fat diet (Wang et al., 2005; Lu et al., 2013; Yan et al., 2015; Wang et al., 2015), little information is available on the effects of dietary carbohydrate on lipid deposition in fish species.

Adipose triglyceride lipase (ATGL) has been proven to selectively hydrolyse triglyceride, the first step of lipolysis, as the rate-limiting lipolytic enzyme (Haemmerle et al., 2006). Recent years, the expression pattern and regulation of ATGL in fish species has been explored with increasingly prominent situation of abnormal lipid deposition (Ji et al., 2012; Wang et al., 2013; Sun et al., 2016a, 2016b). The cDNA sequence of ATGL has been isolated from some fish species, including large yellow croaker, grass carp and javelin goby (Ji et al., 2012; Wang et al., 2013; Huang et al., 2015). In mammals, the expression of ATGL could

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be significantly down-regulated by insulin (Kim et al., 2006; Kershaw et al., 2006; Deiuliis et al., 2008), which appears to be the most potent regulator of ATGL (Watt and Steinberg, 2008). Also, insulin could promote the accumulation of triglyceride (TG) in freshly isolated hepatocytes of yellow catfish (Zhuo et al., 2014), and effectively inhibit adipocyte lipolysis through repressing the expression of ATGL in grass carp (Sun et al., 2017).

FoxO1, belonged to the forkhead transcription factors subfamily, is a central regulator of metabolism (Accili and Arden, 2004). Meanwhile, the promotor region of ATGL possesses two FoxO1-binding sites (Chakrabarti and Kandror, 2009), and FoxO1 could stimulate lipolysis via interacting with its corresponding response elements in the promoter of ATGL in mammals (Chakrabarti and Kandror, 2009, 2011). The activity of FoxO1 could be depressed by insulin through Akt-mediated phosphorylation and nuclear exclusion (Nakae et al., 1999; Calnan and Brunet, 2008; Saltiel and Kahn, 2001). The above finding indicates that lipolysis could be mediated by insulin through the FoxO1/ATGL dependent pathway in mammals. Meanwhile, the repression role of insulin on the expression of FoxO1 has been observed in grass carp (Sun et al., 2017).

The content of plasma insulin increases following a glucose load in fish species (Enes et al., 2011, 2012). Additionally, the adaptation of carnivorous fish to a high carbohydrate diet would improve their insulin response (Mazur et al., 1992), and carbohydrate-rich diet could induce the increase of plasma insulin levels (Párrizas et al., 1994; Banos et al., 1998; Capilla et al., 2003). Previous study in European seabass has demonstrated that the lipid deposition resulted from the intake of high dietary carbohydrate is not attributable to the increase of de novo lipogenesis (Viegas et al., 2016), and therefore, the increase of lipid accumulation maybe partly resulted from the repressed lipolysis through regulating the expression of FoxO1 and ATGL.

The hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) has been largely cultured in Southeast Asia and China due to its fast growth performance and huge potential market value. However, in the aquaculture of this grouper, the increase of lipid deposition in both liver and abdominal adipose tissue caused by the intake of high carbohydrate diet would impact its growth performance and health. Therefore, in the present study, the cDNA sequences of ATGL and FoxO1 gene were isolated, and their gene expression in response to dietary carbohydrate and intraperitoneal insulin injection was investigated. Meanwhile, the promoter of grouper ATGL was cloned and cell transfection and dual-luciferase reporter assays were performed to elucidate the regulation of FoxO1 on ATGL.

2. Material and methods

2.1. Diet preparation

Three isoproteic (52% crude protein) and isolipidic (10% crude lipid) diets were formulated with graded levels of carbohydrate (8.02, 11.89 and 16.08%) (Table 1). Thereinto, brown fish meal, shrimp meal, fermented soybean meal and corn gluten meal were used as major protein sources; Fish oil, soybean oil and soybean phospholipid were used as major lipid sources; and α-starch was used as the main carbohydrate source. All of the dry ingredients were thoroughly mixed after grounded to fine powder through 75 μm mesh. Then, the mixture was thoroughly mixed with the oil mixture (soybean oil and soybean phospholipid), and water was added to produce a stiff dough. The dough was extruded through a 2.5 mm die by a pellet-making machine (KL series, Xinchang Chenshi Mechanical Factory, Zhejiang Province, China). The pellets were post-cooked in the oven at 105 °C for 15 min, and then dried in a ventilated oven at 55 °C. After that, the diets were stored at −20 °C until used.

Table 1
Formulation and proximate analysis of the experimental diets (% dry weight).

Ingredients (/% dry weight)	Dietary carbohydrate content (%)		
	8.02	11.89	16.08
Brown fish meal	47.1	47.1	47.1
Shrimp meal	5.6	5.6	5.6
Fermented soybean meal	7.15	7.15	7.15
Corn gluten meal	6.4	6.4	6.4
Wheat gluten meal	3.5	3.5	3.5
Spray-dried blood meal	2.0	2.0	2.0
Squid viscera meal	1.2	1.2	1.2
Yeast meal	2.0	2.0	2.0
Phospholipid oil	1.5	1.5	1.5
Soybean oil	3.0	3.0	3.0
Monocalcium phosphate	1.0	1.0	1.0
Vitamin premix[a]	1.0	1.0	1.0
Mineral premix[b]	1.0	1.0	1.0
α-starch	3.2	7.4	11.6
Microcystalline cellulose	2.5	2.5	2.5
Zeolite powder	11.85	7.65	3.45
Proximate composition (n = 3)			
Crude protein (%)	52.08	52.33	51.97
Crude lipid (%)	10.37	10.08	10.11
Nitrogen-free extract (%)	8.02	11.89	16.08

[a] Itamin Premix (IU or mg/kg dry diet): vitamin A, 16000 IU; vitamin D_3, 8000 IU; vitamin K_3, 14.72; vitamin B_1, 17.80; vitamin B_2, 48; vitamin B_6, 29.52; vitamin B_{12}, 0.24; vitamin E, 160; vitamin C, 800; niacinamide, 79.20; calcium-pantothenate, 73.60; folic acid, 6.40; biotin, 0.64; inositol, 320; choline chloride, 1500; L-carnitine, 100.

[b] Mineral Premix (mg/kg dry diet): Cu ($CuSO_4$), 2.00; Zn ($ZnS0_4$), 34.4; Mn ($MnS0_4$), 6.20; Fe ($FeS0_4$), 21.10; I ($Ca(IO_3)_2$), 1.63; Se (Na_2SeO_3), 0.18; Co (COC_{12}), 0.24; Mg ($MgSO_4.H_20$), 52.70.

2.2. Animal experiments and sampling

The hybrid grouper used in the present study were obtained from a local fish pond (Hainan, China) and reared in an indoor temperature-controlled re-circulating seawater system (Zhanjiang, China). Then, carbohydrate level study was conducted to investigate the expression of ATGL and FoxO1 in response to dietary carbohydrate, and intraperitoneal insulin study was used to explore the regulation of insulin on the expression of the two above genes. Briefly, for the carbohydrate level study, fish with similar size (initial body weight 21.48 ± 0.24 g) were distributed into 9 tanks (1200 L) at a density of 30 individuals per tank, and each diet was randomly assigned to triplicate groups of fish. The fish were fed to apparent satiation twice daily (8:00 and 16:00) for 8 weeks. At the termination of the feeding trial, blood samples were obtained from the caudal vasculature using 1-mL heparinized syringes after centrifugation (4000 × g, 10 min, 4 °C) for the analysis of insulin content. The liver of six grouper in each tank was then sampled, frozen in liquid nitrogen and stored at −80 °C for gene expression and TG content analysis. As for the intraperitoneal insulin study, triplicate groups of five grouper (80.4 ± 2.1 g) were intraperitoneal injected bovine insulin (5 μg per 100 g fish body mass, sigma, USA), and same volume phosphate-buffered saline (Solarbio, China) was used as control. Liver of three fish in each tank were isolated at 24 h post injection for gene expression analysis. In addition, various tissues (eye, brain, heart, liver, kidney, stomach, intestine and muscle) were isolated from nine grouper individuals to investigate the expression profile of ATGL and FoxO1.

2.3. Insulin and TG content analysis

Plasma insulin content was measured by radioimmunoassay using tilapia insulin as the standard and rabbit anti-tilapia insulin as antiserum according to the method described by Gutiérrez et al. (1984). TG content in liver was analyzed spectrophotochemically at 546 nm